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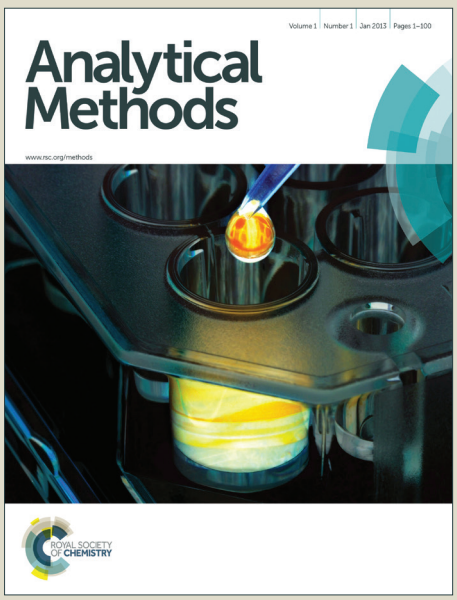

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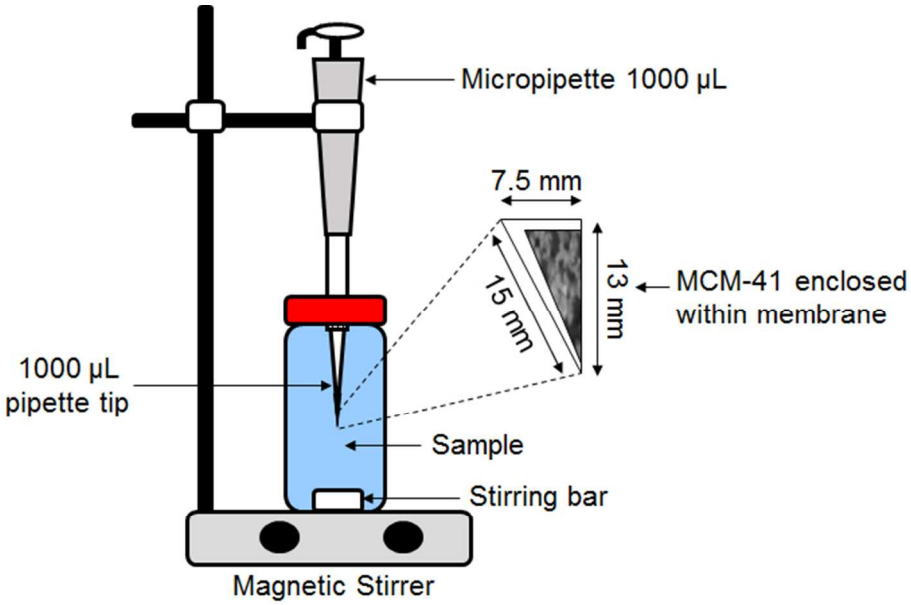

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Solid Phase Membrane Tip Extraction Combined with Liquid Chromatography for the Determination of Azole Antifungal Drugs in Human Plasma

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Abstract

A simple and efficient solid phase membrane tip extraction (SPMTE) was developed using mesoporous silica MCM-41 adsorbent for the determination of three azole antifungal drugs in human plasma prior to high performance liquid chromatography (HPLC). Three azole drugs, namely voriconazole (VRZ), ketoconazole (KTZ) and itraconazole (ITZ) were used as target

analytes. The plasma was deproteinized prior to the extraction using methanol-dichloromethane (75:25, v/v). Optimized extractions were obtained using the following conditions: conditioning solvent, acetone; extraction time, 15 min; desorption time, 15 min; salt addition, 10 % (w/v); pH of sample solution, 8; sample volume, 15 mL and desorption solvent, methanol. A portion of the clean extract (20 µL) was injected into the HPLC-UV system for analysis. Under the optimized conditions, the method demonstrated good linearity with correlation of determination, $r^2 \geq 0.9958$ in the concentration range of 60 - 8000 µg L^{-1} and good limits of detection in the range of 20 - 40 µg L^{-1}. The method showed satisfactory precisions with RSDs <16 % (n = 3) and high relative recoveries in the range of 82.5 - 111.0 %. The MCM-41-SPMTE method proved to be simple, efficient, and requires minimal amounts of organic solvent that supported the green chemistry concept.

Keywords: Sample preparation, Solid phase membrane tip extraction, MCM-41, Azole antifungal drugs, human plasma

1. Introduction

The compression of body's immune system during therapeutic treatments such as organ transplantation, use of immunosuppressive agents in cancer treatment, or diseases such as acquired immunodeficiency syndrome (AIDS) urge the occurrence of mycosis in humans [1]. The other cause of mycosis is the improper use of broad spectrum antibiotics that reduce the bacterial population which commonly competing with fungi. Therefore, the development of antifungal drugs are crucial in order to find agents which arrive at the infection focus [2]. Voriconazole (VRZ) and itraconazole (ITZ) are drugs belonging to a group of antifungal

compounds called triazole while ketoconazole (KTZ) belongs to the imidazole group. These are systemic azole drugs (medicines taken orally or by injection). The chemical structure, protein binding %, partition coefficient (Log *P*) and dissociation constant of a solution (pK_a) [3] for each azole antifungal drug are described in Table 1. The quantification of plasma concentrations of VRZ, KTZ and ITZ is very important to determine toxicological profile and drug tolerance in humans. Previous clinical studies proposed that plasma VRZ concentrations of >6 μg mL^{-1} were associated with occasional liver function abnormalities [4]. KTZ is an azole drug that is commonly used for systemic and local infections [5]. Clinical studies suggested that azole may participate in interaction with many drugs in the event of substantial amounts of the residues in the human body [6]. Therefore, a fast, simple, accurate and inexpensive analytical method for the monitoring of antifungal azole drugs in human plasma is crucial to provide the association between drug concentration and response.

Several analytical instruments have been used for the analysis of azole antifungal drugs including liquid chromatography [7-9], gas chromatography [10] and capillary electrophoresis [11, 12]. The most common methods for the determination of azole antifungal drugs in water and biological samples were high performance liquid chromatography (HPLC) coupled with mass spectrometry [13, 14] and HPLC with ultraviolet detector [15, 16]. However, identification and quantification of targeted drugs in complex matrices may be difficult due to high levels of interferences and lower detection ability of instrument. Thus, the development of appropriate sample preparation is required to eliminate major interferences and to concentrate the target analytes in the complex matrices prior to the final instrumental analysis [17].

Numerous sample preparation methods have been developed for the analysis of azole antifungal drugs in biological, environmental and formulations samples including liquid-liquid extraction (LLE) [18] and solid phase extraction (SPE) [19-22]. LLE has drawbacks of time consuming, labour intensive and requires large volumes of organic solvents. SPE has significant improvement over LLE, but it is relatively expensive. The development of microextraction methods for azole drugs such as ultrasound-enhanced surfactant-assisted dispersive liquid-liquid microextraction (UESA-DLLME) [5], liquid phase microextraction (LPME) [16] and solidification of floating organic drop dispersive liquid-liquid microextraction (SFODME) [23] have greatly reduced the organic solvent consumption and produced less waste. Recently-introduced solid phase membrane tip extraction (SPMTE) is an interesting microextraction method due to its advantages in terms of simplicity, low solvent usage, easy to use and low analysis cost. SPMTE has been successfully applied to the determination of atrazine herbicides [24] and organochlorine and pyrethroid pesticides [25] in water samples.

Since discovered by Mobil researchers in 1992 [26], mesoporous silica, MCM-41 has drawn great interests due to its special characteristics of large surface area (>1000 m^2 g^{-1}), uniform pore structure (20 - 50 nm) and huge pore volume (>0.7 cm^3 g^{-1}) [27]. MCM-41 offered promising applications in catalysis [28], sensor design [29], drug delivery [30] and separation techniques [31]. Due to its special characteristics and strong adsorption ability, MCM-41 and organo-functionalized MCM-41 have been successfully used as the solid phase microextraction (SPME) coating for the analysis of polycyclic aromatic hydrocarbons (PAHs) [32-34]. More recently, MCM-41 has been used as adsorbent for the determination of PAHs in gaseous samples [35].

Nevertheless, the development of microextraction methods to extract azole antifungal drugs in biological sample is still limited.

In the present study, MCM-41 was employed as adsorbent in SPMTE for the extraction of selected azole antifungal drugs in human plasma followed by HPLC with ultraviolet (HPLC-UV) detection. This method provided a simple and efficient microextraction means for the determination of selected azole drugs in human plasma.

2. Experimental

2.1 Chemicals and reagents

Tetraethyl orthosilicate (TEOS) and cetyltrimethylammonium bromide (CTABr) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonia solution (NH_4OH) (28 %) and hydrochloric acid (36 %) were obtained from QRëC Asia (Selangor, Malaysia). Potassium dihydrogen phosphate, sodium hydroxide, dichloromethane, acetone, isopropanol and acetic acid were purchased from Merck (Darmstadt, Germany). All chemicals were of analytical-reagent grade. HPLC grade methanol and acetonitrile were obtained from J.T. Baker (Pennsylvania, USA). Deionized water of 18.2 MΩ was purified by Nano ultrapure water system (Barnstead, USA). Analytical grade sodium chloride (NaCl) was purchased from Bendosen (Selangor, Malaysia). Voriconazole (>95 %) was obtained from Clearsynth (Mumbai, India) while ketoconazole (98 %) and itraconazole (98 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock standard solutions (1000 μg mL^{-1}) of the azole drugs were prepared in

methanol and were stored in the freezer at -4°C. Q3/2 Accurel 2E HF (R/P) polypropylene (PP) membranes (157 μm thickness, 0.2 μm pore size) were obtained from Membrana (Wuppertal, Germany).

2.2 Sample Collection and Pretreatment

Human plasma samples were obtained from Penang General Hospital (Penang, Malaysia) and stored at -4 °C prior to use. The frozen samples were thawed in water at room temperature (25°C) before use. The thawed samples were vortexed to ensure complete mixing of the contents. The plasma sample (3 mL) was transferred into a 15-mL centrifuge tube and spiked with the mixed standard solutions at different concentrations. After adding 500 μL of 100 mM potassium dihydrogen phosphate buffer (pH 8), the solution was vortexed to ensure uniform mixing. The solution was added with 3 mL of methanol-dichloromethane (75:25, v/v) and centrifuged for 10 min at 6000 rpm. The resulting supernatant (approximately 7 mL) was added with 10 % (w/v) of NaCl and diluted with distilled water to 15 mL.

2.3 Preparation of MCM-41-SPMTE tip

The SPMTE procedure was adopted from a previously reported work [24] with slight modification on the type of adsorbent. In brief, the SPMTE device consisted of MCM-41 enclosed in PP membrane attached to 1000.0 μL pipette tip. MCM-41 was synthesized using the molar gel composition and synthesis conditions as reported previously [36]. A PP sheet membrane was cut into an equilateral triangle with each side of approximately 15 mm. The edge

of the membrane was folded to form a scalene triangle shape with sides of 15, 13 and 7.5 mm. The edge of the longest flap was then heat-sealed using a portable sealer. A portion (3 mg) of MCM-41 was added into the cone-shaped membrane and the open edge was then heal-sealed to secure the adsorbents. Each tip was cleaned in acetone for 10 min and then stored in the same solvent until use. The 1000.0 μL pipette tip end was cut-off approximately 7.0 mm length and the cone-shaped membrane was inserted into the end of the modified tip. The schematic of MCM-41-SPMTE is shown in Fig. 1.

2.4 MCM-41-SPMTE Procedure

Solid phase membrane tip was placed in 15 mL of diluted human plasma that was stirred at 1000 rpm. A dynamic extraction procedure was carried out at 5 min interval of the extraction. A 600 μL of aqueous sample was withdrawn into the tip at constant low speed using a digital micropipette (Eppendorf, Germany). After a dwelling time of approximately 3 s, the withdrawn aqueous sample was released from the tip back into the sample vial at constant low speed. This procedure was repeated for 5 times and the micropipette was detached from the tip. The dynamic extraction procedure was repeated at every 5 min interval until the end of extraction. After extraction, the cone-shaped membrane was removed, rinsed in ultrapure water, dried with lint-free tissue and placed in a 500 μL safe-lock tube. The analytes were desorbed by ultrasonication for 15 min in methanol (100 μL) and 20 μL of the solution was injected into the HPLC system.

2.5 Chromatographic Conditions

The HPLC system (Waters LC) consisted of a pump, a Rheodyne 3699 injector, sample injection valve with a 20-µL internal loop for sample introduction, and a UV detector (Shimadzu, Japan) was used for chromatographic analysis. The separation was performed on a 5.0 µm Zorbax SB-C_{18} column (4.6 µm I.D. × 100 mm) (Agilent Technology, USA) and the mobile phase was a mixture of 10 mM phosphate buffer (adjusted to pH 6.5 with 1 M potassium hydroxide)-acetonitrile (35:65, v/v) at a flow rate of 1.0 mL min^{-1}. Detection was monitored at 254 nm.

3. Results and Discussion

3.1 Optimization of MCM-41-SPMTE

In order to optimize the extraction, seven parameters namely conditioning solvent, extraction time, sample pH, salt addition, sample volume, desorption solvent and desorption time were investigated.

3.1.1 Conditioning Solvents and Extraction Time

The conditioning solvent was used to activate the hydrophobic nature of PP membrane containing MCM-41 by immersing the membrane in the organic solvents for 2 min prior to the microextraction. In the present study, several organic solvents, namely methanol, acetonitrile, isopropanol, acetone and dichloromethane were tested as conditioning solvent in MCM-41-SPMTE.It was found that acetone gave the highest peak areas response for VRZ and KTZ, while, ITZ showed the highest peak area when dichloromethane was used as conditioning solvent (Fig. 2). As compared to dichloromethane, acetone showed increased peak areas of about 173 % and

24 %, for VRZ and KTZ, respectively, while for ITZ, the peak area was slightly decreased by only about 10 %. Therefore, acetone was used as conditioning solvent in subsequent analyses to activate the PP membrane containing MCM-41 adsorbent and to ensure the reproducible retention of analytes during the extraction process.

As mass transfer is a time-dependent process, the effect of extraction time was investigated in the range of 5 to 20 min. In SPMTE, the extraction-time profile of target analytes is important to configure the time after which equilibrium is achieved in the system. It was found that there was a rapid increase in extraction efficiency of all analytes when the extraction time was prolonged from 5 to 15 min and the peak areas remained nearly constant or slightly decreased when the extraction time was further increased (20 min) (Fig. 3). This phenomenon might be due to the back-extraction of analytes from adsorbent into sample solution [24]. Thus, 15 min was selected as the optimum extraction time and used in further experiments.

3.1.2 Sample pH, Salt Addition and Sample Volume

Sample pH is one of the most important parameters in the extraction process. The studied azole antifungal drugs possess weak base moiety and can be present in water in both ionized and neutral forms. The extraction of analytes in their neutral form is expected to be easier than when they exist in ionized form. Since VRZ, KTZ and ITZ are weak base azole drugs with a pK_a values from 2.27-12.71 (Table 1), the pH of the solution was varied from pH 7.0 to 8.5 in order to assemble the neutral form of analytes to assist the extraction. The best extraction efficiency

was obtained at pH 8 which gave the highest peak areas that corresponded to the pK_a values of all analytes (VRZ, KTZ and ITZ). Therefore, pH 8.0 was chosen and used in further analyses.

The addition of NaCl may change the ionic strength and the solubility of analytes in the sample solution. The effect of salt addition on the extraction efficiency was evaluated by the addition of NaCl from 0 to 25 % (w/v) into the sample solution. The results showed an increase of peak area response for two of the azole drugs (VRZ and ITZ) from 0 to 10 % (w/v) of NaCl addition, but KTZ showed the highest peak area at 5 % (w/v) of NaCl addition (Fig. 4). At 10 % NaCl addition, peak area of KTZ slightly decreased by about 2.5%, while peak areas of VRZ and ITZ showed an increase of about 33 % and 26 %, respectively. However, thereafter, the peak areas of all analytes dropped when 15 % of NaCl was added into the sample solution due to the high viscosity of sample solution which decreased the diffusion rate of the analytes [37]. Therefore, to compromise the efficiency for all analytes, 10 % (w/v) of NaCl was used for all subsequent experiments.

Sample volume was used to determine the adsorption capacity of MCM-41 after the equilibrium was attained. The results showed that the optimum sample volume was at 15 mL which gave highest peak area responses for VRZ and ITZ. However, in case of KTZ, highest peak area was obtained when 10 mL of sample solution was applied in the extraction (Fig. 5). Based on the results obtained at 15 mL sample volume, peak areas of VRZ and ITZ increased by about 19 % and 2 %, respectively, while peak area of KTZ decreased by about 4 %. The drop in peak area response for all analytes was observed when larger sample volume (20 mL) was applied in the extraction probably due to the saturation of MCM-41 (~ 3 mg) capacity for a larger sample

volume [24]. To compromise the extraction performance for all analytes, sample volume of 15 mL was used in subsequent experiments.

3.1.3 Desorption Solvent, Desorption Time and Volume of Desorption Solvent

In this study, the choice of the desorption solvent relies on its compatibility with the liquid chromatographic system. The organic solvents with different polarity indices, namely methanol (5.1), acetonitrile (5.8), isopropanol (3.9) and tetrahydrofuran (4.0) were examined [38]. Due to the azole's relatively polar properties, a polar solvent should give better results than a less polar solvent. It was found that methanol gave the highest peak areas for KTZ and ITZ, while acetonitrile and methanol showed similar peak areas for VRZ (Fig.6). Thus, in order to obtain the best extraction performance for all studied analytes, methanol was used as desorption solvent for all subsequent analyses.

The desorption time is the time that is required to desorb all the analytes from the MCM-41 in the extraction tip. In order to evaluate the effect of desorption time, the PP tip was ultrasonicated in different durations in the range of 5 to 20 min. It was found that the maximum desorption was achieved within 15 min. Beyond this point (20 min), the desorption efficiency of all analytes decreased probably due to the analytes being re-adsorbed by the adsorbent (Fig. 7) [39]. Thus, 15 min desorption time was used for subsequent experiments. Desorption volume of 100 µL was used as that is the lowest volume to fully submerge the PP tip.

3.2 Method Validation

Under the optimum conditions, the proposed method was validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), precision and recovery. The MCM-41-SPMTE validation data obtained are shown in Table 2. Linear calibration curve was obtained by plotting different concentrations of spiked plasma sample ranging from 60 to 8000 μg L^{-1}. Good linearity was observed for SPMTE method with correlation of determination, $r^2 \geq 0.9958$ for all target analytes. LODs were calculated as three times the signal-to-noise ratio (*S/N* * 3) and LOQs measured as ten times the signal-to-noise ratio (*S/N* * 10). The LODs and LOQs for azole drugs were in the range of 20 - 40 μg L^{-1} and 60 - 100 μg L^{-1}, respectively.

Intra- and inter-day precisions were determined at low, medium and high concentrations (100, 2000, 8000 μg L^{-1}) with triplicate analyses on the same day and over three different days using plasma sample, respectively. The results showed acceptable relative standard deviations (RSDs) ranging from 2.6 to 9.0 % (n = 3) and 8.0 to 15.4 % (n = 3) for intra- and inter-day precision, respectively (Table 3), signifying the good precision of the developed method.

3.3 Analysis of Human Plasma Samples

In initial experiments, no azole drugs were detected in the human plasma samples. Therefore, in order to assess the usefulness of the method, different concentrations of analytes were spiked in the sample. Relative recoveries of the method were calculated based on the percentage ratio between the concentration found in the sample and concentration spiked in the same sample of each analyte. The proposed MCM-41-SPMTE method that was conducted under the optimum conditions provided high relative recoveries in the range of 82.5 - 111.0 % for azole drugs at

different concentrations (low, medium and high) (Table 3). Chromatograms of drug-free plasma and spiked plasma (Fig. 8) indicated a clean sample pretreatment provided by the proposed method thus suitable for quantification of the concentrations of selected drugs in human plasma samples.

3.4 Comparison with Other Reported Methods

The analytical characteristics of the proposed MCM-41-SPMTE method were compared with other reported methods (Table 4**).** LPME [16] and SFODME [23] methods resulted in excellent sensitivity for azole drugs, however, it requires relatively longer extraction time to reach equilibrium for each analysis (≥35 min). The LOD, precision and recovery of MCM-41 were comparable to other reported liquid chromatography method [18, 21, 22]. The use of 3 mg of adsorbent and organic solvent (100 µL) have added incentives in MCM-41-SPMTE as an alternative microextraction method for azole drugs. Furthermore, MCM-41-SPMTE has the advantage of simplified analytical extraction process without sacrificing high recoveries by using simple devices and ultrasonication system.

4. Conclusions

The present study has successfully developed a simple, and efficient microextraction method of azole antifungal drugs in human plasma. Parameters that affected the extraction efficiency including conditioning solvent, extraction time, sample pH, salt addition, sample volume, desorption solvent and desorption time were examined thoroughly throughout the study. MCM-

41-SPMTE method showed acceptable sensitivity with satisfactory precision and recoveries of azole drugs in human plasma. The use of only small amounts of adsorbent and minute amounts solvent have added great consideration for MCM-41-SPMTE to be an alternative "green" microextraction methods for azole drugs determination in human plasma.

Acknowledgements

The authors wish to thank Universiti Teknologi Malaysia and the Ministry of Higher Education Malaysia (MOHE) for facilitation and financial support through research grant number QJ130000.2526.03H79 and studentship for N. Yahaya. Gifts of human plasma samples from Prof. Y. Hassan and Prof. N. A. Aziz (Universiti Teknologi Mara, Malaysia) are gratefully acknowledged.

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Figure captions:

Fig. 1: Schematic of MCM-41-SPMTE system.

Fig. 2: Effect of conditioning solvent on MCM-41-SPMTE of azole antifungal drugs (n = 3 in each case). Error bars represent the standard deviations.

Fig. 3: Effect of extraction time on MCM-41-SPMTE of azole antifungal drugs (n = 3 in each case). Error bars represent the standard deviations.

Fig. 4: Effect of salt addition on MCM-41-SPMTE of azole antifungal drugs (n = 3 in each case). Error bars represent the standard deviations.

Fig. 5: Effect of sample volume on MCM-41-SPMTE of azole antifungal drugs (n = 3 in each case). Error bars represent the standard deviations.

Fig. 6: Effect of desorption solvent on MCM-41-SPMTE of azole antifungal drugs (n =3 in each case). Error bars represent the standard deviations.

Fig. 7: Effect of desorption time on MCM-41-SPMTE of azole antifungal drugs (n =3 in each case). Error bars represent the standard deviations.

Fig. 8: Chromatograms obtained from MCM-41-SPMTE of blank azole drugs and spiked azole drugs at 100 and 2000 ppb.

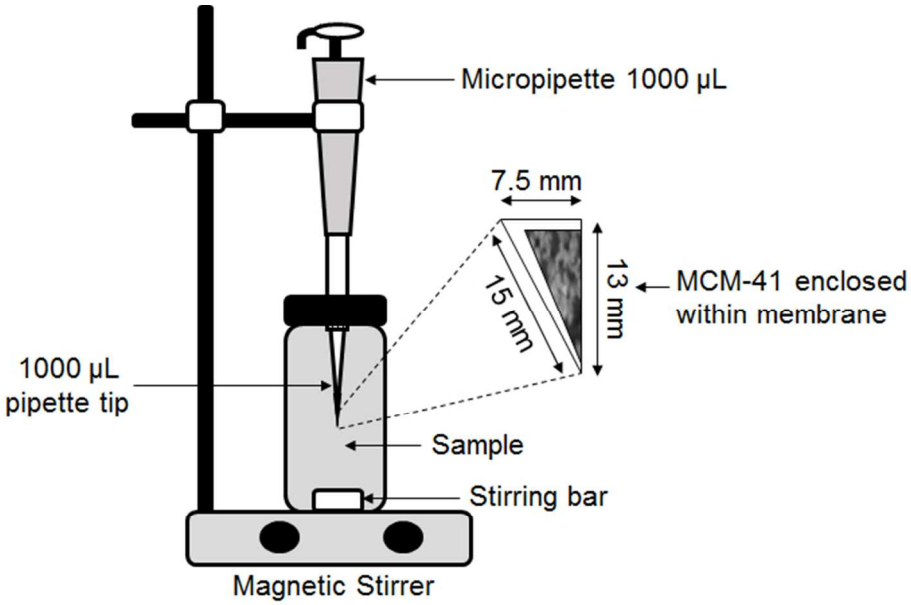
Table captions:

Table 1. Chemical structure, protein binding (%), Log P, pK_a of voriconazole (VRZ), ketoconazole (KTZ) and itraconazole (ITZ).

Table 2. MCM-41-SPMTE validation data.

Table 3. Precisions and relative recoveries of MCM-41-SPMTE of spiked human plasma samples.

Table 4. Comparison of analytical performances of azole antifungal drugs in biological fluids.



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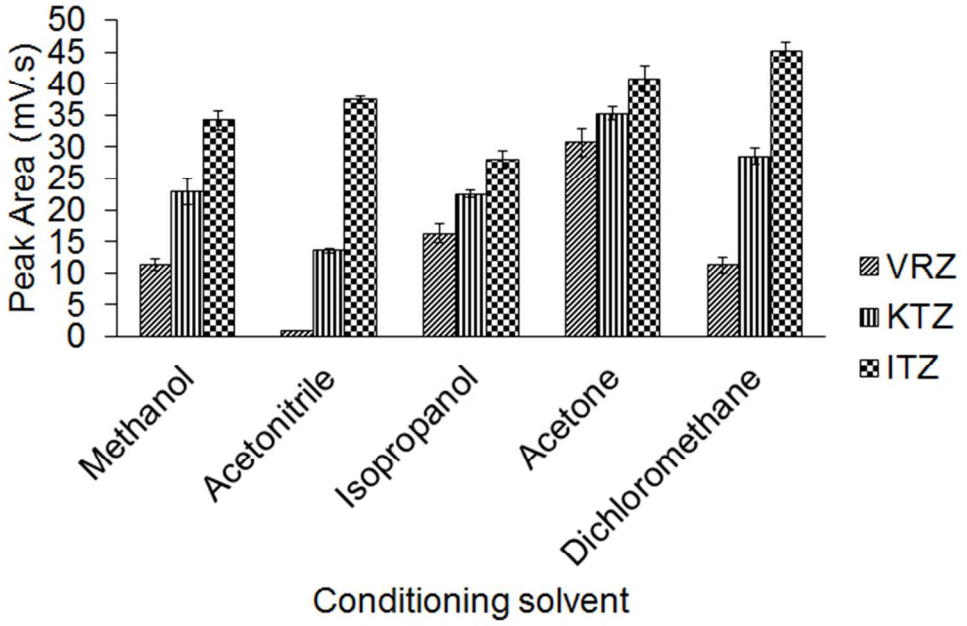


Fig. 2: Effect of conditioning solvent on MCM-41-SPMTE of azole antifungal drugs (n = 3 in each case). Error bars represent the standard deviations.
180x118mm (96 x 96 DPI)

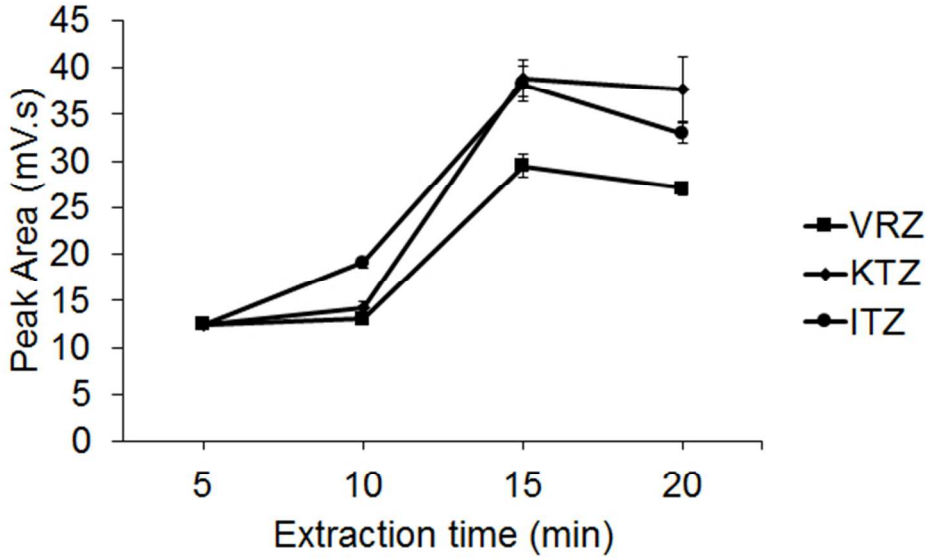


Fig. 3: Effect of extraction time on MCM-41-SPMTE of azole antifungal drugs (n = 3 in each case). Error bars represent the standard deviations.
170x98mm (96 x 96 DPI)

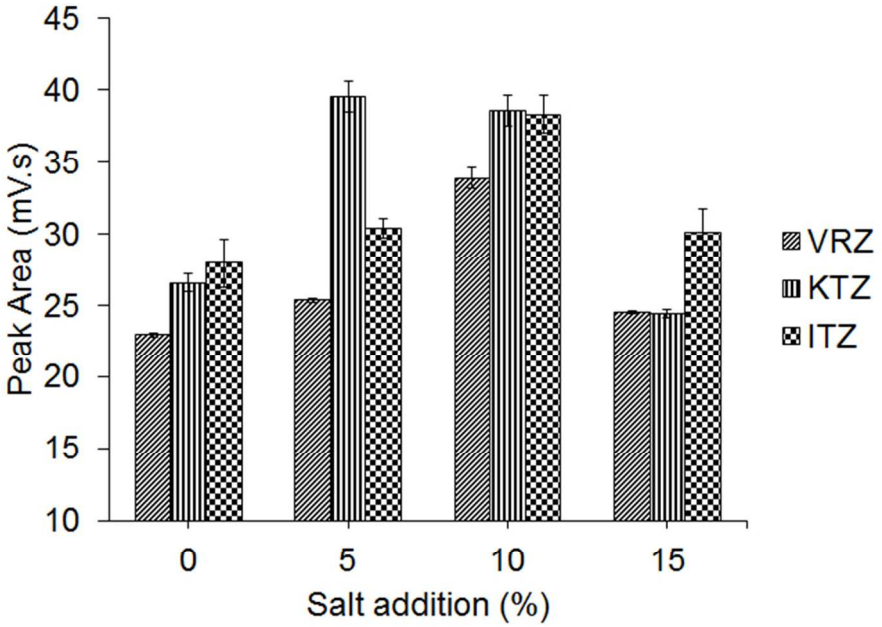


Fig. 4: Effect of salt addition on MCM-41-SPMTE of azole antifungal drugs (n = 3 in each case). Error bars represent the standard deviations.
191x131mm (96 x 96 DPI)

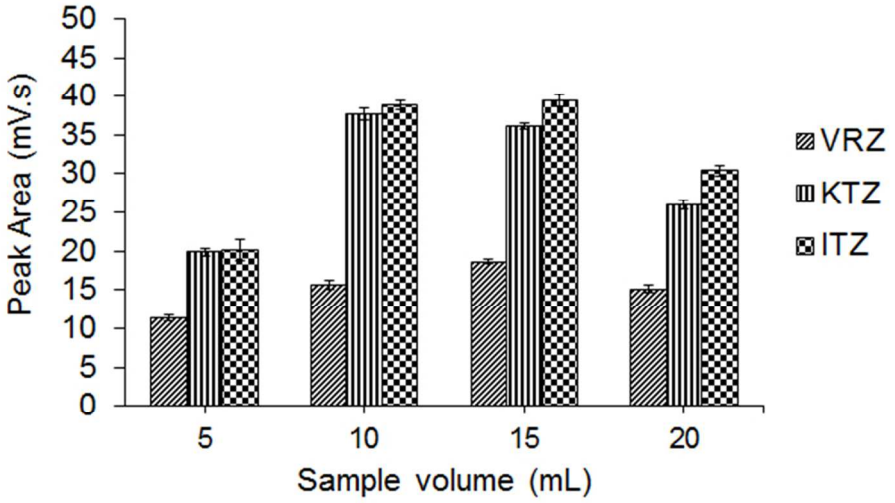


Fig. 5: Effect of sample volume on MCM-41-SPMTE of azole antifungal drugs (n = 3 in each case). Error bars represent the standard deviations.
175x92mm (96 x 96 DPI)

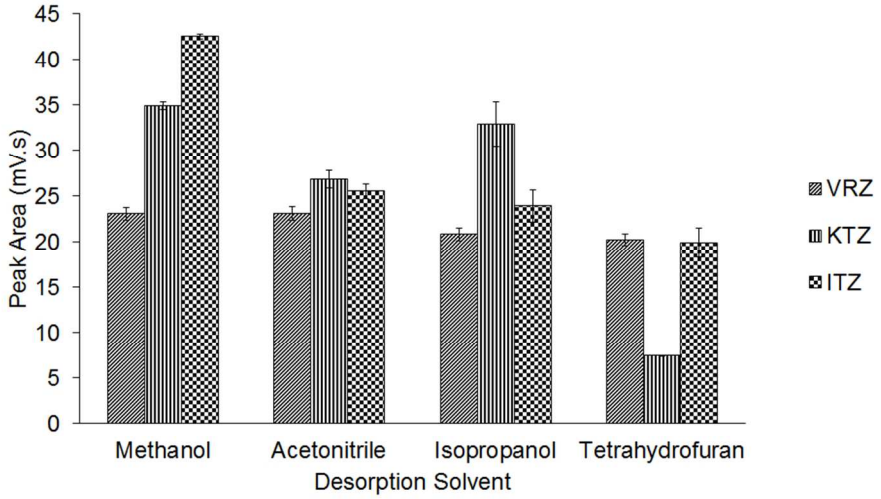


Fig. 6: Effect of desorption solvent on MCM-41-SPMTE of azole antifungal drugs (n =3 in each case). Error bars represent the standard deviations.
273x152mm (96 x 96 DPI)

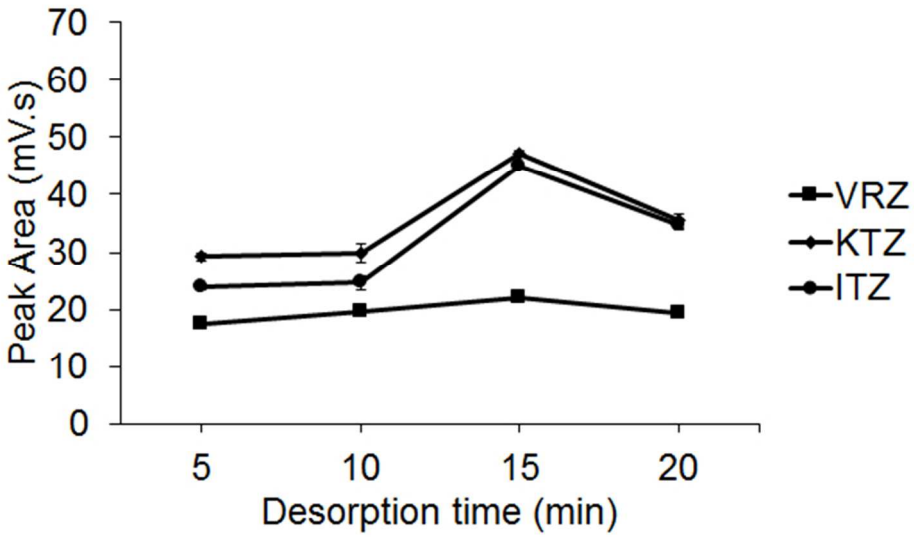


Fig. 7: Effect of desorption time on MCM-41-SPMTE of azole antifungal drugs (n =3 in each case). Error bars represent the standard deviations.
170x95mm (96 x 96 DPI)

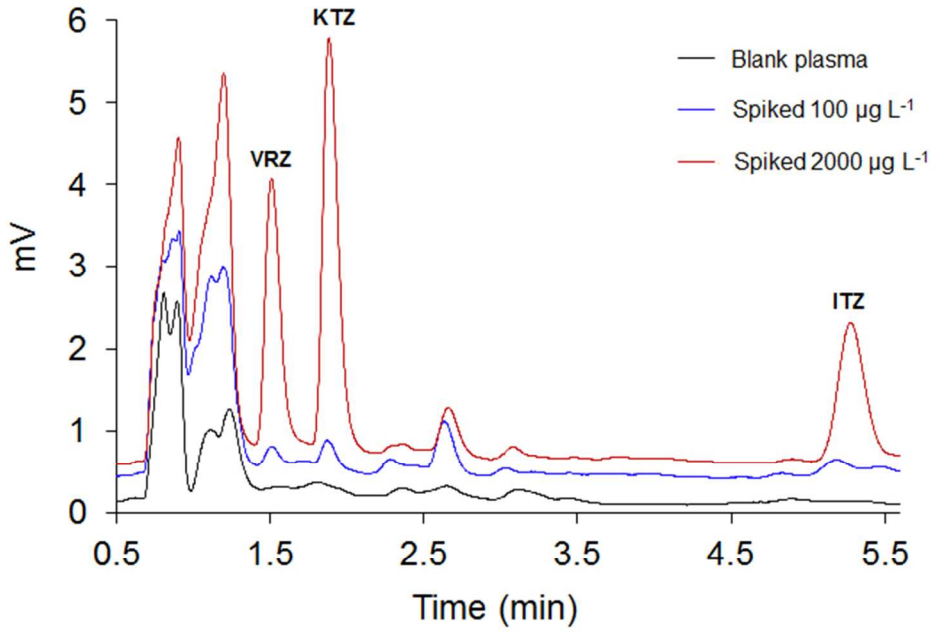
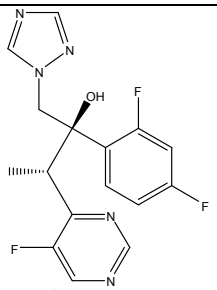
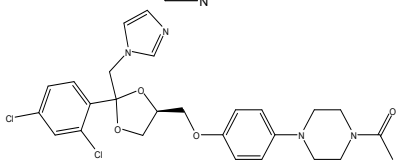
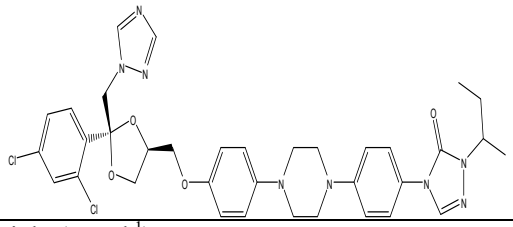


Fig. 8: Chromatograms obtained from MCM-41-SPMTE of blank azole drugs and spiked azole drugs at 100 and 2000 ppb.
183x122mm (96 x 96 DPI)

Table 1

Chemical structure, protein binding (%), Log *P*, pK_a of voriconazole (VRZ), ketoconazole (KTZ) and itraconazole (ITZ)

Analytes	Chemical Structure	Protein binding (%)	Log *P*	pK_a
VRZ		58[b]	1.0[b]	2.27, 12.71[b]
KTZ		84-99[b]	4.0[b]	2.9, 6.5[c]
ITZ		100[b]	6.5[b]	3.7[b]

[a] Molecular weight (g mol^{-1})
[b] http://www.drugbank.ca (accessed on 4th September 2013)
[c] Ref. [3]

Table 2

MCM-41-SPMTE validation data

Analytes	Linearity ($\mu g\ L^{-1}$)	RSD (%)	LOD ($\mu g\ L^{-1}$)	LOQ ($\mu g\ L^{-1}$)	Correlation of determination (r^2)
VRZ	90- 8000	≤ 8.2	30	90	0.9958
KTZ	60 - 8000	≤ 8.9	20	60	0.9991
ITZ	100 - 8000	≤ 7.1	40	100	0.9994

Table 3

Precisions and recoveries of MCM-41-SPMTE of spiked human plasma samples

Analytes/ Spiked human plasma ($\mu g\ L^{-1}$)	Recovery of MCM-41-SPMTE %, (% RSD, n = 3)			Intra-day precision (% RSD, n = 3)			Inter-day precision (% RSD, n = 3)		
	100	2000	8000	100	2000	8000	100	2000	8000
VRZ	95.2 (2.5)	111.0 (2.3)	99.6 (3.7)	7.2	5.8	2.6	15.4	8.0	14.2
KTZ	110.9 (4.1)	105.6 (2.2)	88.5 (5.8)	4.2	9.0	4.0	14.8	10.1	8.2
ITZ	92.4 (10.7)	100.9 (7.9)	82.5 (3.9)	4.7	3.5	5.6	10.7	8.0	11.3

Table 4:

Comparison of analytical performances of azole antifungal drugs in biological fluids

Instrument	Analyte(s)	Matrices	Sample preparation	Linear range ($\mu g\ L^{-1}$)	LOD ($\mu g\ L^{-1}$)	LOQ ($\mu g\ L^{-1}$)	Precision (% RSD)	Recovery (%)	Ref.
HPLC-UV	VRZ, KTZ, ITZ	Human plasma	MCM-41-SPMTE	50 - 8000	20 - 40	60 - 100	2.6 - 7.2 (n = 3)	82.5 - 111.0	Proposed work
	VRZ, KTZ, ITZ	Human plasma	SPE	50 - 40000	20 - 50	50 - 150	1.51 - 11.66 (n = 24)	93.8 - 106.7	[22]
	KTZ	Urine, Plasma	LPME	5.0 - 500	0.9	-	9.1 - 11.2 (n = 3)	95.6 - 97.6	[16]
	VRZ	Serum and plasma	LLE	100 - 20000	30	100	< 10 (n = 5)	89.6	[21]
HPLC-PDA	KTZ	Plasma, Urine	SFODME	0.1 - 200	0.014	-	4.7 - 8.6 (n = 5)	93.6 - 98.15	[23]
HPLC-MS/MS	VRZ	Plasma	LLE	500 - 10000	60	130	2.8 - 3.5 (n = 10)	97.8 – 109.0	[18]

Abbreviations:
HPLC-UV- High performance liquid chromatography- ultra violet detector
HPLC-PDA- High performance liquid chromatography- photodiode array detector
HPLC-MS/MS- High performance liquid chromatography- tandem mass spectrometry
SPE- Solid phase extraction
LPME- Liquid phase microextraction
LLE- Liquid-liquid extraction
SFODME- Solidification of floating organic drop microextraction
MCM-41-SPMTE- Mesoporous silica, MCM-41 solid phase membrane tip extraction